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(21) International Application Number: PCT/SE99/02095 (22) International Filing Date: 16 November 1999 (16.11.99) (30) Priority Data: 9803958-9 19 November 1998 (19.11.98) SE (71) Applicant (for all designated States except US): PHARMACIA & UPJOHN AB [SE/SE]; S-112 87 Stockholm (SE). (72) Inventors; and (75) Inventors/Applicants (for US only): JAMES, Stephen [GB/SE]; Björnmossenavägen 27, S-162 45 Vällingby (SE). DAHLMAN-WRIGHT, Karin [SE/SE]; Thespisvägen 10, S-167 71 Bromma (SE). LAKE, Staffan [SE/SE]; Farkostvägen 8, S-181 35 Lidingö (SE). BUTCHER, Steve [GB/SE]; Svampvägen 9, S-191 52 Sollentuna (SE). CLIMENT, Isabel [ES/SE]; Odengatan 70, S-113 22 Stockholm (SE). (74) Agents: TANNERFELDT, Agneta et al.; Pharmacia & Upjohn AB, S-112 87 Stockholm (SE).		(81) Designated States: AU, CA, JP, NO, NZ, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: METHOD FOR SCREENING FOR SUBSTANCES WHICH ARE ACTIVATORS, INHIBITORS OR BINDERS OF PKB BY THE USE OF A SUBSTRATE PEPTIDE AND THE USE OF THE SUBSTRATE PEPTIDE (57) Abstract The invention relates to a method for screening for substances which are activators or inhibitors of Protein kinase B (PKB) and can be used as a kinase substrate for PKB. The substrate is a peptide comprising the two sequences R-R-R-A-A/V-S-M and R-P/S-R-S/T-S/N-S-N and preferably comprising the three sequences R-P-R-S-C-T-W, R-R-R-A-A/V-S-M and R-P/S-R-S/T-S/N-S-N. These sequences as well as the human Afx, amino acids 12-505 or amino acids 1-505, can be used in assays measuring the activity of PKB, and in screening for substances which are activators or inhibitors of gene transcriptional regulation of forkhead proteins through the catalytic activities of PKB. These sequences can also be used for discrimination between the effects of compounds which mediate insulin action through transcription from those which modulate activity of enzymes involved in metabolism by phosphorylation.		

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Method for screening for substances which are activators, inhibitors or binders of
5 PKB by the use of a substrate peptide and the use of the substrate peptide

Summary

The present invention relates to a method for screening for substances which are
activators or inhibitors of Protein kinase B (PKB) and can be used as a kinase substrate
10 for PKB. The substrate is a peptide comprising the two sequences R-R-R-A-A/V-S-M
and R-P/S-R-S/T-S/N-S-N and preferably comprising the three sequences R-P-R-S-C-
T-W, R-R-R-A-A/V-S-M and R-P/S-R-S/T-S/N-S-N.

These sequences as well as the human Afx , amino acids 12-505 or amino acids 1-505,
can be used in assays measuring the activity of PKB, and in screening for substances
15 which are activators or inhibitors of gene transcriptional regulation of forkhead
proteins through the catalytic activities of PKB. These sequences can also be used for
discrimination between the effects of compounds which mediate insulin action through
transcription from those which modulate activity of enzymes involved in metabolism
by phosphorylation.

20

Background

The mechanistic basis by which insulin regulates the disposition of glucose by animals
has been elucidated in close detail in recent years. A cascade of interacting proteins has
been described which, when functioning in the normal situation, serves to transduce
25 the signals emanating from insulin, causing cells of different origin to take up glucose
from the bloodstream and store it (White 1997). In the current understanding,
activation of the insulin receptor by insulin causes the phosphorylation and activation
of insulin receptor substrate (IRS) proteins. These serve to act as docking proteins for a
variety of downstream proteins leading to their activation. A key downstream protein
30 in insulin signalling is phosphoinositide 3-kinase (PI3K) which catalyses the
production of the second messenger phosphatidylinositol 3,4,5-trisphosphate. This is a

lipid and is central to the activation of PKB ((Franke et al. 1995); (James et al. 1996); (Franke et al. 1997); (Klippel et al. 1997); (Alessi et al. 1997); (Stokoe et al. 1997)). It is bound by the pleckstrin homology (PH) domains of PKB and of an upstream kinase called 3-phosphoinositide-dependent kinase 1 (PDK1) which is involved in the
5 activation of PKB.

PKB appears to be a key intermediary in the regulation of glucose utilisation and control of protein synthesis by insulin (Cross et al. 1995); (Cohen et al. 1997); (Peak et al. 1998); (Gingras et al. 1998)). Thus, it has been demonstrated to phosphorylate and
10 inactivate glycogen synthase kinase 3 (GSK3; (Cross et al. 1995)), permitting the synthesis of glycogen from glucose. Furthermore, in cardiac myocytes, PKB has been shown to phosphorylate and activate phosphofructo kinase-2 (Deprez et al. 1997) whose product, fructose 2,6-bisphosphate, acts as an allosteric activator of glycolysis. A third likely substrate for PKB is the type 3B cyclic AMP phosphodiesterase
15 (Wijkander et al. 1998), which in insulin-responsive tissues is activated by phosphorylation, leading to the inactivation of adrenergic-stimulated processes.

Although the range of substrates phosphorylated by PKB is diverse, all of them described share a common short primary sequence which serves as the target region for
20 PKB. The first identified consensus sequence of amino acids was in GSK-3, described as gly-arg-pro-arg-thr-ser-ser-phe-ala-glu-gly (GRPRTSSFAEG) (Cross et al. 1995). See also WO 97/22360.

Comparison with other substrates allows a consensus sequence to be derived from this which is likely to contain the essential features for phosphorylation by PKB. This
25 consensus is: RXRXXS/TF (Alessi et al. 1996) where F is phenylalanine but could be replaced by another bulky hydrophobic residue. Data in the literature (not only Alessi et al. 1996 but also Walker et al. 1998) strongly suggest that the amino acid sequence for phosphorylation by PKB must include a large hydrophobic residue directly C-terminal to the phosphorylation site. Such a residue has thus been described as crucial
30 for phosphorylation by PKB.

Studies in *C. elegans* have recently suggested that an important effect of insulin may be to suppress the transcriptional activity in the family of transcription factors (Ogg et al. 1997). By inactivating a specific transcription factor in *C. elegans* (daf-16), the authors have shown that development in *C. elegans* can bypass a modulated insulin
5 receptor signalling pathway.

We have previously shown and claimed (see patent application SE9801530-8) that PKB phosphorylates a peptide sequence derived from the transcription factor *forkhead FKHR*. This is a member of a subfamily of the wider forkhead superfamily, which is
10 related to the *C. elegans* nematode worm forkhead protein daf-16.
However, in SE9801530-8 we disclosed peptides which do not include a large hydrophobic residue directly C-terminal to the phosphorylation site and we demonstrated that these peptides are similarly good substrates for PKB as the earlier known peptides with the large hydrophobic residue directly C-terminal to the
15 phosphorylation site.

Figure 1 shows an alignment of the daf-16-related fork head proteins taken from the literature and we refer here to Parry, P et al, Genes Chromosomes Cancer 11 (2), 79-84 (1994) and Borkhardt, A et al., Oncogene 14 (2), 195-202 (1997) when relating to
20 Afx and to Anderson, M.J et al., Genomics 47 (2), 187-199 (1998) and Galili, N et al., Nat.Genet. 5 (3), 230-235 (1993), when relating to FKHR, SEQ ID No 8

The sequence and its close homologues disclosed in SE9801530-8 is underlined.

Afx, SEQ ID No 7 is a human homologue of daf-16.

Paradis and Ruvkun in Genes & Development, 12:2488-98, 1998 show that two *C.*
25 *elegans* Akt/PKB homologues, akt-1 and akt-2, transduce insulin receptor-like signalling in *C. elegans* and discuss the target of Akt/PKB signalling as a transcription factor.

The questions whether PKB phosphorylates the intact forkhead protein, whether it
30 does this in an insulin-sensitive manner and what effect this has on the activity if the

forkhead protein itself, i.e. does insulin regulate forkhead transcriptional activity *via* PKB, are not answered by the earlier work on Afx, daf-16, parts of FKHR or PKB. If Afx could be inhibited, such an inhibitor may be of great use in the metabolic therapeutic area, such as in the treatment of metabolic syndrome, insulin resistance and insulin resistance related diseases such as diabetes type 2.

Figures

Figure 1. Alignment of forkhead proteins

Figure 2. Phosphorylation of human recombinant Afx by recombinant PKB

10 Figure 3a to 3d. The effect of insulin on phosphorylation of Afx12-505 by PKB

Figure 4A and 4 B. Expression constructs

Figure 4C. Relative alkaline phosphatase activities, Example 3

Figure 5. Relative alkaline phosphatase activities, Example 4

15 *The invention*

We have earlier (see SE9801530-8) found that PKB α activity is stimulated to phosphorylate a small peptide. Our earlier data (patent applied for) also suggest that we know where in the protein PKB acts to add phosphate, namely within the consensus sequences for PKB action. The small peptides can be useful in screening methods.

20 But for a eukaryote cell based screening, the function of the whole protein is of importance.

We have now found that we can also use a substrate peptide which comprises the phosphorylation sites of FKHR, FKHRL1 and Afx, (residues 19-26, 251-257 and 314-321 (FKHR), 27-33, 248-254 and 310-316 (FKHRL1), 27-33, 192-198 and 257-263 (Afx, our numbering from our genomic clone). Afx has accession number X939996.

The sequences, included in the family of forkhead protein FKHR, SEQ ID No 8, FKHRL1, SEQ ID No 9 and preferably Afx, SEQ ID No 7, have the potential to be a

target for regulation by PKB and this has been supported by *in vitro* experiments that show that the recombinant protein can be phosphorylated by PKB.

Our data show that phosphorylation of Afx protein by PKB is greater when the PKB activity is isolated from cells which have been stimulated with insulin or IGF-1 than in
5 cells which are not stimulated. These data suggest that insulin and IGF-1 primary messengers stimulate PKB activity against a variety of intracellular targets, including the forkhead proteins. This is the first time that this has been shown in a mammalian cellular system.

Afx has thus been shown as good substrate for PKB and can therefore be used in
10 screening for substances which are activators, inhibitors or binders of PKB. When screening for substances which are activators or inhibitors of PKB itself, it is important to know if the substance stimulates or inhibits PKB in a cell and especially in an insulin-stimulated cell. This can only be measured when a substrate is used which includes most of the primary sequence of the fork head protein.

15 Assays which measure the functional activity of Afx can be used for discrimination between the effects of compounds which mediate insulin action through transcriptional regulation, from those compounds which modulate activity of key metabolic enzymes by phosphorylation. Gene transcriptional regulation involves activation or repression of the transcription of the enzymes and other components involved in metabolism, for
20 example, repression of phosphoenolpyruvate carboxykinase (PEPCK) in liver.

Afx can be used in the search for new substrates for PKB as templates for sequence searches and/or for primers in techniques of molecular biology such as PCR.

By PKB any isoform thereof is included.

The invention is defined in the attached claims.

25

Example 1. Phosphorylation of human recombinant Afx by recombinant PKB.

GST-PKB pre-coupled to sepharose beads was activated by incubation with lysate (approx. 200µg/ml) from IGF-1-stimulated H9C2 cardiac myocytes in the presence of unlabelled ATP and phosphatase inhibitors. After thorough washing of the beads to
30 ensure that contamination with cell lysate was minimal, beads were incubated with approximately 5µg recombinant human Afx (comprising amino acids 12-505) termed

Afx12-505 or truncated Afx comprising amino acids 86-236, termed Afx86-236, which we hypothesise still contains a PKB phosphorylation site, in the presence of radioactive ATP.

Pre-activated PKB was incubated with Afx proteins (, lanes 1-4, Afx12-505; lanes 5-6
5 Afx86-236 in Figure 2) in the presence of radioactive ATP and proteins were subsequently resolved by SDS-PAGE. In lanes 3-6, kinase-permissive conditions were used and each Afx protein was phosphorylated. Control reactions included a buffer which does not support kinase activity (lanes 1 and 2 236 in Figure 2) and omission of PKB (lanes 7-10 236 in Figure 2). Incubations were stopped after 30 min, proteins
10 were separated by SDS-PAGE, using a 4-20% gradient gel, and after drying, the gel was exposed by autoradiography.

Results. Figure 2 shows that in reactions containing both PKB and Afx (12-505 or 86-236) performed in a buffer which supports kinase reactions (50mM Tris/HCl pH 7.4, 0.1mM EDTA, 0.1mM EGTA, 10mM MgCl₂ plus various protease and phosphatase
15 inhibitors), both the full length (lanes 3 and 4) and truncated forms (lanes 5 and 6) of the transcription factor become phosphorylated. When PKB was omitted from the reactions, neither protein became labelled with radioactive phosphate (lanes 7-10) indicating that phosphorylation was not due to an endogenous contaminating kinase activity. Furthermore, reactions between PKB and Afx were precluded in a buffer
20 which does not support a kinase reaction (lanes 1 and 2, performed in a buffer containing Tris/HCl pH 7.5, high NaCl and 1M imidazole). Thus, recombinant forkhead transcription factor does appear to be a substrate for PKB *in vitro*.

Example 2. The effect of insulin on phosphorylation of Afx12-505 by PKB

25 To address whether insulin stimulation of cells leads to increased phosphorylation of Afx12-505 by PKB, quiescent L6 muscle cells were pre-treated with rapamycin and PD-98059 to inhibit activation of p70S6kinase and MAP kinase, respectively, and stimulated with 100nM insulin for 5 min. Cells were lysed and lysates from resting and insulin-stimulated cells were resolved using MonoQ anion exchange
30 chromatography, as has been previously described by Walker *et al* (Walker, K.S. (1998) *Biochem. J* 331, 299-308). Three discrete peaks of PKB activity were observed,

identified by measuring phosphorylation of the PKB substrate peptide (RPRTSSF), which is a known sequence exactly as described by Walker *et al* (1998) (data not shown). The first peak was PKB γ , whilst peaks 2 and 3 contained PKB α (identified by Western blotting, data not shown).

- 5 Each Mono Q fraction was analysed for the ability to phosphorylate Afx12-505. 1 μ g recombinant Afx12-505 was incubated with 10 μ l each fraction in the presence of radioactive ATP and 10 μ M unlabelled ATP for 1 hour at 37°C. Reactions were terminated by addition of SDS reducing buffer, and after boiling, samples were resolved by 10% SDS-PAGE. Gels were stained with coomassie stain (Figure 3a),
- 10 which revealed a single band at the expected molecular weight for Afx12-505, dried and exposed on the phosphorimager for 24 hours. The gel in Figure 3a is representative of four gels where reactions from each reaction were separated. Images from the phosphorimager showed that after incubation with samples from individual Mono Q fractions which contained PKB activities in the presence of radioactive ATP
- 15 as described above, the major Afx12-505 band was radioactively labelled, along with two minor contaminants. Fraction numbers are shown at the top of respective lanes and the position of Afx12-505 is indicated. Figure 3b1: fractions from insulin-stimulated cells; Figure 3b2: fractions from control resting cells.
- Result: Labelling was greater when Afx12-505 was incubated with column fractions
- 20 from insulin-stimulated cell lysates. It is clear from figure 3b1 and 3b2 that cells which were stimulated with insulin have higher activity than cells without insulin.
- Bands corresponding to Afx12-505 from each lane in Figure 3b were quantitated using the phosphorimager and the extent of phosphorylation is shown using the arbitrary pixel units of the software. Open symbols in Figure 3c shows insulin-stimulated cells;
- 25 closed symbols in Figure 3c shows resting cells.
- Figure 3c shows that radioactive labelling of Afx12-505 by column fractions from non-stimulated L6 cells remained fairly constant across the column, with minor peaks observed. However, for insulin-stimulated lysates, phosphorylation of Afx12-505 fell into three distinct peaks, corresponding with the peaks of PKB activity.

Identical experiments as in Example 2, Figure 3c, but for a separate experiment in which PKB from lysates of resting L6 cells was compared with IGF-1-stimulated cells, show that the peaks of PKB α , (peaks 2 and 3 on Mono Q column) activity are stimulated to phosphorylate Afx compared with activities in unstimulated cells, whereas the activity of the γ -isoform of PKB is similar in both cell lysates (Figure 3d). These data show both insulin and IGF-1 lead to the activation of PKB isoforms whose activity against Afx *in vitro* is markedly enhanced. Thus, stimulation of cells with these agonists may lead to phosphorylation of Afx by PKB, demonstrating a novel regulation of transcription by PKB.

Example 3, Afx enhances transcription of an IRE driven reporter gene

The ability of Afx12-505 to activate an IRE (insulin responsive enhancer) DNA sequence was investigated by transient transfection assays in HepG2 cells.

HepG2 cells were transfected with 1.5 μ g expression plasmid, 0.5 μ g reporter plasmid

and 1 μ g RSV LUC (Control for transfection efficiency) using CaPO₄ method. Cells were grown in MEM with 10% FBS. Differences in transfection efficiency are corrected for by measured Luciferase activity. Transfections have been performed in triplicate and one representative experiment is shown. The expression and reporter plasmids are shown in Figure 4A. The sequence of the IRE, which is derived from the IGFBP-1 gene, is shown in Figure 4B.

Relative alkaline phosphatase activities 48 hr. after transfection are shown in Figure 4C. This shows that transfection of Afx12-505 specifically induces the alkaline phosphatase reporter gene when an IRE DNA sequence is inserted upstream (Compare columns 4 and 3, respectively). Some IRE specific activity is seen when the cells are transfected with the pEGFP-C3 plasmid, lacking Afx sequences (columns 1 and 2, respectively). This is presumably due to endogenous transcription factors activating via this DNA sequence in HepG2 cells. Furthermore, expression of Axf86-282 induces no additional alkaline phosphatase activity above that seen with pEGFP-C3.

Example 4. The ability of Afx to activate transcription is suppressed by insulin

The ability of insulin to suppress the Afx mediated stimulation of the IRE driven reporter gene was investigated by culturing HepG2 cells transfected with an expression plasmid which expresses Afx12-505 and a reporter plasmid with an IRE driven alkaline phosphatase reporter gene in serum free medium in the presence and absence of insulin. HepG2 cells were transfected with 1.5 µg expression plasmid expressing Afx12-505 and 0.5 µg reporter plasmid pSEAP2-IRE using CaPO₄. Cells were grown in MEM with 10% FBS. 24 hr. after transfection the medium was changed to serum free medium with (see Figure 5, left column) and without (see Figure 5, right column) 100nM Insulin. Relative alkaline phosphatase activities 48 hr. after the change to serum free medium are shown.

Figure 5 shows that the ability of Afx12-505 to activate the IRE driven reporter gene is markedly suppressed in the presence of insulin (left column).

The mechanism by which insulin suppress the Afx mediated stimulation of the IRE driven reporter gene was investigated by culturing HepG2 cells transfected with an expression plasmid which expresses Afx12-505, a reporter plasmid with an IRE driven alkaline phosphatase reporter gene and a plasmid which expresses full length human PKB α . Control transfections included the vector alone, into which the PKB α gene had been inserted. HepG2 cells were transfected with 1 µg expression plasmid expressing Afx12-505, 0.5 µg reporter plasmid pSEAP2-IRE, 1 µg PKB α expressing or control plasmid and 1 µg RSV LUC (Control for transfection efficiency) using a CaPO₄ method. Cells were grown in MEM with 10% FBS. 24 hours after transfection the medium was changed to serum free medium with and without 100 ng/ml insulin. Differences in transfection efficiency are corrected for by measured Luciferase activity. Relative alkaline phosphatase activities (%) 48 hours after the change to serum free medium are shown (Figure 6). These data show that the ability of Afx12-505 to activate an IRE driven reporter gene is markedly suppressed by overexpressed PKB and that the remaining activity is further suppressed by insulin. This is genetic evidence for the direct regulation of Afx by PKB, which we infer from our biochemical data (where PKB phosphorylates Afx protein directly).

We have demonstrated (data not shown) that Afx alone allowed yeast cells to grow, after transfection with Afx constructs, under non-permissive conditions. Thus, fusion constructs, consisting of a heterologous DNA-binding domain, e.g. the DNA-binding domain from the yeast transcription factor Gal4, fused to Afx12-505, Afx233-505 or Afx283-505 act as strong transcriptional activators of genes including appropriate regulatory sequences, e.g. Gal4 response elements. This can be exploited in screens to search for inhibitors of Afx transactivation function. These data show that Afx is a strong transcriptional activator in yeast cells and allows yeast cells to grow in hostile conditions. Therefore, we could use Afx-transfected yeast cells as a screen for Afx inhibitors. Successful hits would be detected because no cells would be able to grow. A screening method for compounds which are inhibitors of Afx could thus be by determining cell number in the presence of a test compound and a reference compound. The compound which reduces growth would be an inhibitor of Afx.

15.

One function of Afx is thus to activate genes which are normally repressed by insulin and on these genes insulin acts to suppress the transcriptional activation of Afx. The experiments shown in Figures 4C and 5 support this hypothesis.

20 Discussion.

Our initial prediction that the family of forkhead proteins related to FKHR may be a target for regulation by PKB has been supported by *in vitro* experiments that show recombinant protein can be phosphorylated by PKB. Our earlier data (patent applied for) also suggest that we know where in the protein PKB acts to add phosphate, namely within the consensus sequences for PKB action, shown in Figure 1.

Our data also show that phosphorylation of Afx proteins by PKB is greater when the PKB activity is isolated from cells which have been stimulated with insulin or IGF-1, than in cells which are not stimulated. These data suggest that insulin and IGF-1 primary messengers stimulate PKB activity against a variety of intracellular targets, including the forkhead proteins. This is the first time that this has been shown in a

mammalian cellular system. Thus we can construct a signal transduction cascade from the insulin or IGF-I receptor all the way via a family of transcription factors to a DNA response element regulating genes involved in the metabolic response.

- 5 Our prediction is that PKB-mediated phosphorylation of the forkhead proteins may act to inhibit the activity of the transcription factor against certain genes (e.g. PEPCK), whilst perhaps increasing the transcription of other genes involved in the metabolic response.
- 10 Afx peptide can be used for finding compounds useful for treating patients having a dysfunction of essential components in metabolism such as transducers of the insulin signalling pathway and enzymes involved in metabolism. The compounds found from the claimed screening are anticipated also to be used against long term complications resulting from insulin resistance, such as vascular dysfunction, loss of neuronal cells,
- 15 retinopathy and beta-cells in pancreas.

These compounds are not possible to find by using the modulators as described in WO 97/22360.

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SEQUENCE LISTING

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Trp Pro Leu Gln Arg Pro Glu Leu Gln Ala Ser Pro Ala Lys Pro Ser
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Gly Glu Thr Ala Ala Asp Ser Met Ile Pro Glu Glu Glu Asp Asp Glu
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Gly Gly Gly Gly Ser Gly Thr Leu Gly Ser Gly Leu Leu Leu Glu Asp
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Ser Ala Arg Val Leu Ala Pro Gly Gly Gln Asp Pro Gly Ser Gly Pro
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Asn Phe Asp Ser Leu Ile Ser Thr Gln Asn Val Val Gly Leu Asn Val
645 650 655

Gly Asn Phe Thr Gly Ala Lys Gln Ala Ser Ser Gln Ser Trp Val Pro
660 665 670

Gly

CLAIMS

5

1. Method for screening for substances which are activators, inhibitors or binders of Protein kinase B (PKB) by the use of a substrate peptide comprising two sequences selected from SEQ ID No 1 and SEQ ID No 2; SEQ ID No 3 and SEQ ID No 4 and SEQ ID No 1 and SEQ ID No 5.

10

2. Method for screening according to claim 1 in which the substrate peptide comprises the three sequences selected from SEQ ID No 6, SEQ ID No 1 and SEQ ID No 2; SEQ ID No 6, SEQ ID No 3 and SEQ ID No 4 or SEQ ID No 6, SEQ ID No 1 and SEQ ID No 5.

15

3. Method for screening according to claim 2 in which the substrate peptide comprises the three sequences SEQ ID No 6, SEQ ID No 1 and SEQ ID No 5.

4. Method for screening according to any of claim 1 to 3 in which the substrate peptide is forkhead protein FKHRL1, FKHR or Afx from amino acid number 12.

5. Method for screening according to any of claim 1 to 3 in which the substrate peptide is the forkhead protein Afx 1- 501, SEQ ID No 7.

6. Method for screening for substances which are activators, inhibitors and binders of gene transcriptional regulation by forkhead proteins through the catalytic activities of PKB characterised by the use of any of the substrate peptide according to any of claims 1 - 4.

7. Method for screening for substances which have the capacity to modulate PKB activity characterised by the use of any of the substrate peptide according to any of claims 1 - 4.
- 5 8. Method for identifying a compound that activates PKB in vitro, comprising:
- a) contacting a test compound with the substrate peptide according to any of claims 1 - 4 for a time sufficient to form a complex with the substrate peptide
 - b) detecting the complex by detecting the phosphorylation in the complex, so that if a complex is detected a compound that activates PKB is identified.
- 10 9. Method for aiding in the identification of a compound for use in treating metabolic disease comprising the steps of
- a) incubating a cell in the presence and absence of a test compound, wherein said cell has been altered to contain a nucleic acid molecule comprising an insulin response element operable linked to a reporter gene and said cell expresses a
 - 15 wild-type Afx protein;
 - b) determining the level of expression of said reporter gene in said cell incubated in the presence and absence of said test compound; and
 - c) selecting a compound that induces a higher level of expression of said reporter
 - 20 gene in step b) than that expressed in the absence of said compound, for use in treating metabolic disease.
10. Use of a substrate peptide according to any of claims 1 - 4 in assays measuring the activity of PKB.
- 25 11. Use of a substrate peptide according to any of claims 1 - 4 in screening for substances which are activators or inhibitors of gene transcriptional regulation by forkhead proteins through the catalytic activities of PKB.
- 30 12. Use of substrate peptide according to any of claims 1 - 4 for discrimination between the effects of compounds which mediate insulin action through

transcription from those which modulate activity of enzymes involved in metabolism by phosphorylation.

13. Use according to claim 9 for discrimination between the effects of compounds
5 which mediate insulin action through transcription via forkhead transcription factor family from those which modulate activity of enzymes involved in metabolism by phosphorylation.
14. A method for making a pharmaceutical formulation for the treatment of disease,
10 comprising
- a) contacting a test compound with the substrate peptide according to any of claims 1 to 4 for a time sufficient to form a complex with the substrate peptide
 - b) detecting the complex by detecting the phosphorylation in the complex, so that if a complex is detected a compound that activates PKB is identified.
 - 15 c) manufacturing bulk quantities of the compound identified in step b) and
 - d) formulating the compound manufactured in step c) in a pharmaceutically acceptable carrier.

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```

# Consensus          #...P...          #.#-.-#EP..R.R^
FKHRL1  -----MAEAPASPAPLSPLEVELDPEFEPQSRPRS
FKHR     -----MAEAPQV-----VEIDPDFEPLPRPRS
daf16    MMEMLVDQGTDASSSASTSTSSVSRFGADTFMNTPDD-----VMMNDMEPIPRDRC
Afx      -----MRIQPQKAAAI-----IDLDPDFEPQSRPRS

# Consensus  -TWP#.RP-#.          #--...#-
FKHRL1  CTWPLQRPQLQ-----ASPAKPSGETAADSMIPEEEDDEDDEDGGGRAGSAMAIGGGGG
FKHR     CTWPLPRPEFSQSN SATSSPA-PSGSAAAN-----PDAAAGLPSASAAAVSADFM
daf16    NTWPMRRPQLEP-----PLNSSPIIHEQIPEED-----ADLYGSNEQ
Afx      CTWPLPRPEIAN-----QPSEPPEVEPDLGEK-----

# Consensus          .....#...P-          .....^          .....
FKHRL1  SGTLGSGLLLED SARVLAPGGQDPGSGPATAA---GGLSGGTQ---ALLQPQQLPPPO
FKHR     SNL---SLLEESDFPQAPGSVAAAVAAAAAAATGGLCGDFQGPEAGCLHPAPPQPPPP
daf16    CGQLGGASSNGSTAMLHTPDGNSHQTSFSPDFRMSSEPDDTVSG-----
Afx      -----VHTEGRSEPILLPS-----RLSEPAGGPQPGILGAVTGP-----

# Consensus          + ---RRNAWGN.SYA-#I-.AI.^#-KRLTL^Q#YE
FKHRL1  -----PGA-AG-SGQPRK-CSSRRNAWGNLSYADLITRAIESSPDKRLTLSQIYE
FKHR     GPVSQHPPVPVPAAGPLAGQPRKSSSSRRNAWGNLSYADLITKAIESSAEKRLTLSQIYE
daf16    -----KKTTRRNAWGNMSYAE LITTAIMASPEKRLTLAQIYE
Afx      -----RKGSSRRNAWGNQSYAEFISQAIESAPEKRLTLAQIYE

# Consensus  WMV.-VPYF+DKGDSNSSAGWKNSIRHNLSLHS+F#+#-NE^GKSSWW#NP- ^K.G+
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FKHR     WMVKSVPYF+DKGDSNSSAGWKNSIRHNLSLHSKFIRVQNEGTGKSSWWMLNPEGGKSGK
daf16    WMVQNVPYFRDKGDSNSSAGWKNSIRHNLSLHSRFRVQNEGAGKSSWWVINPD-AKPGR
Afx      WMVRTVPYF+DKGDSNSSAGWKNSIRHNLSLHSKFIVHNEATGKSSWWMLNPEGGKSGK

# Consensus  .PRR  R^.-#---K...+^+...KK .#..... ^...-# ^.....S.-
FKHRL1  APRR---RAVSMDNSNKYTKSRGRAAKKKAALQTAPE--SADDSP-SQLSKWPGSPTSRS
FKHR     SPRR---RAASMDNNSKFAKRSRAAKKASLQSGQE--GAGDSPGSQFSKWPASPGSHS
daf16    NPRRTRERSNTIETTTKAQLEKSRGAKK-RIKERALMGSLHSTLNGNSIAGSIQTISHD
Afx      APRR---RAASMDSSSKLLRGRSKAPKKKPSVLPAPPEGATPTSPVGHFAGWSGSPCSRN
          |-----|

# Consensus  ...D          #--FR.R--SN#S  -.S-R#SP#.  .....
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FKHR     NDDFDN-----WSTFRPTSSNAS--TISGRLSPIM--TEQDDLGEED--VHSMVYP
daf16    LYDDDSMQGAFDNPSSFRPTQSNLSIPGSSSRVSPAIGSDIYDDLE-----
Afx      REEAD-----MWTTFRPRSSSNAS--SVSTRLSPLRPESEVLAE-----

# Consensus          .#^          #P.          D.#-.....
FKHRL1  SSASLSPSVSKPCTVELPRLTDMAGTMNLNDGLTENLMDLLDNITLPPSQPSPT-----
FKHR     PSAAKMAST-----LPSLSEISNPEN-----MENLLDNLLNLSSTSLTVSTQS
daf16    -----FPSWVGESVPAIPS-----DIVDRDQMRIDATTHIG
Afx      -----IPASVSSYAGGVPT-----LNEGL-----ELLDGLNLTSSHSLLSRSGLS

```

Figure 1

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```

# Consensus      ....
FKHRL1          --GGLMQRSSSPYTTKGSGLSPTSSFNSTVFGPSSLNSLRQSPMQTIQENKPATFSSM
FKHR            SPGTMMQQTPCYSFAPPNTSLNSPSPNYQKYTYGQSSMSPLPQMPIQTLQDNK-SSYGGM
daf16           G-----VQIK-----
Afx            G-----FSLQHPGVTGPLHTYSSSLFSPA-----EGPLSAG

# Consensus      ....#.T-.#...-#-.....^Q....
FKHRL1          SHYGNQ--TLQDLLTSD-SLSHSDVMMTQSDPLMSQASTAVSAQNSRRNVMLRNDPMMSF
FKHR            SQYNCA PGLLKELLTSD-SPPHNDI-MTPVDPGVAQPNRVLGQNV---MMGPNSVMSTY
daf16           -----QESKPIKTEPIAPPPSYHELNSVRGSCAQNP LL-----
Afx            ECGFSSSQALEALLTSDTPPPPADVLMTQVDPILSQAPTLLLLLGG L-----

# Consensus      .~.##.          ~.....#L.....-..-#.#.....P#.
FKHRL1          AAQPNQGS LVN-QNLLHHQHQTQGALGGSRALNSVSNMGLSES-SSLGSAKHQQQSPVS
FKHR            GSQASHNKMMNPSSHTHPGHAQQTSAVNGRPLPHTVSTMPTSGMNRLTQVKTPVQVPLP
daf16           -----RNPIVP-----STNFKPMPLPGAYGNY-QNGGITPINWLSTSNSSPLP
Afx            ---PSSSKLAT-----GVGLCPKPLEARGPSS-LVPTLSMIAPPPVMASAPIP

# Consensus      ....-..#.....^~          --.#P.DL-          .....D#-.#I.-.
FKHRL1          QSMQTLSDSLSGSSLYSTSANLPVMG---HEKFPSDDL---DMFNGLSLECDMESIIRSE
FKHR            HPMQM--SALGGYSSVSSCNGYGRMGLLHQEKLPDDL---GMFIERLDCDMESIIRND
daf16           GIQSC--GIVAAQHTVAS-----SSALPIDLENLTLPDQPLMDTMDVDALIRHE
Afx            KALGT--PVLTPPTEAAS-----QDRMPQDLD---LDMYMENLECDMDNIISDL

# Consensus      #..^---.-#-F-.
FKHRL1          LMDADGLDFNFDLSLISTQNVVGLNVGNFTGAKQASSQS WVP
FKHR            LMDGDTLDFNFDNVLPNQ-----SFPHSVKTTHSWVSG
daf16           LSQAGGQHIFDL-----
Afx            MDEGEGLDFNFEPDP-----

```

Figure 1 Cont.

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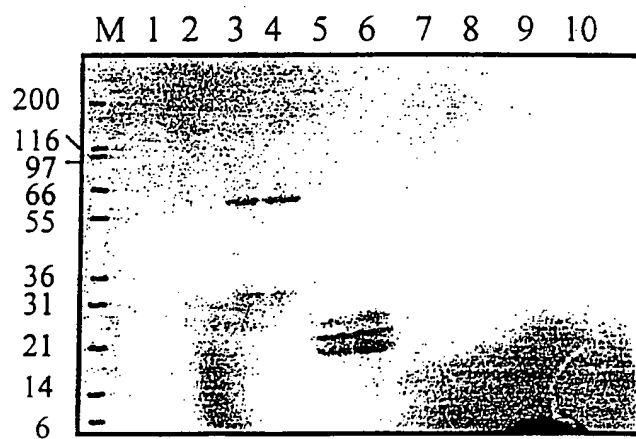


Figure 2

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Figure 3a

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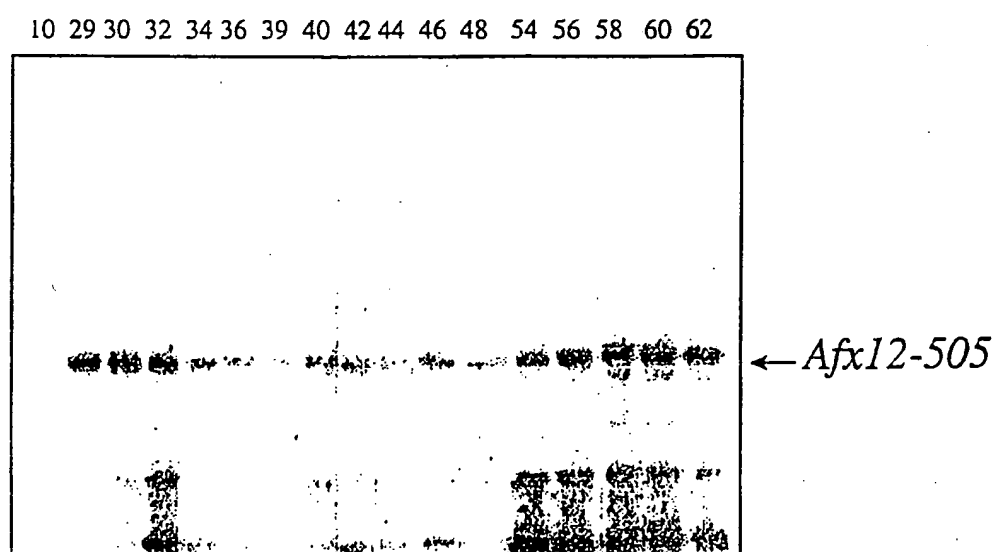


Figure 3b1

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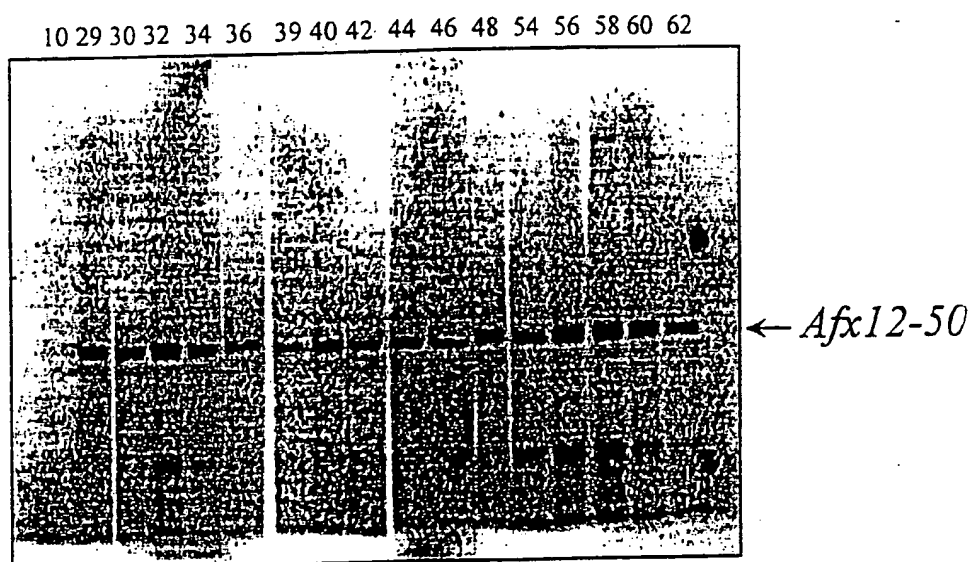


Figure 3b2

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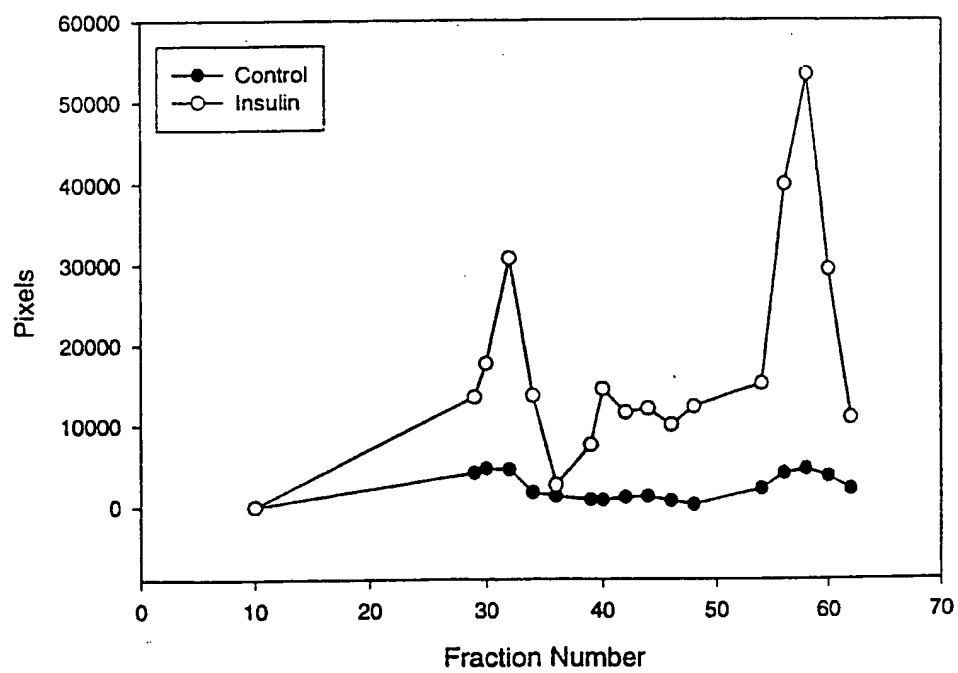


Figure 3c

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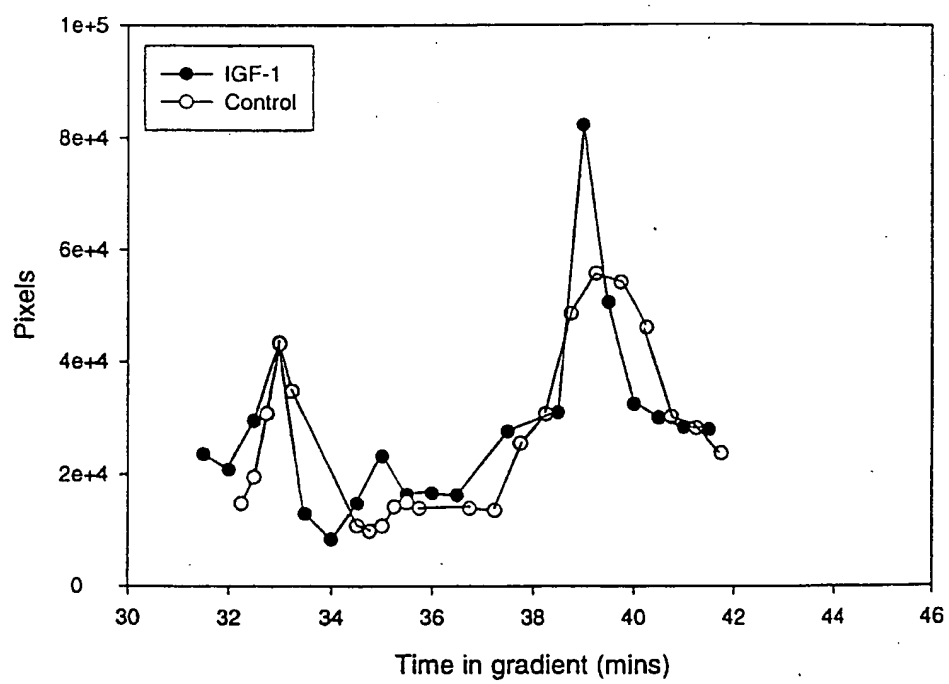


Figure 3d

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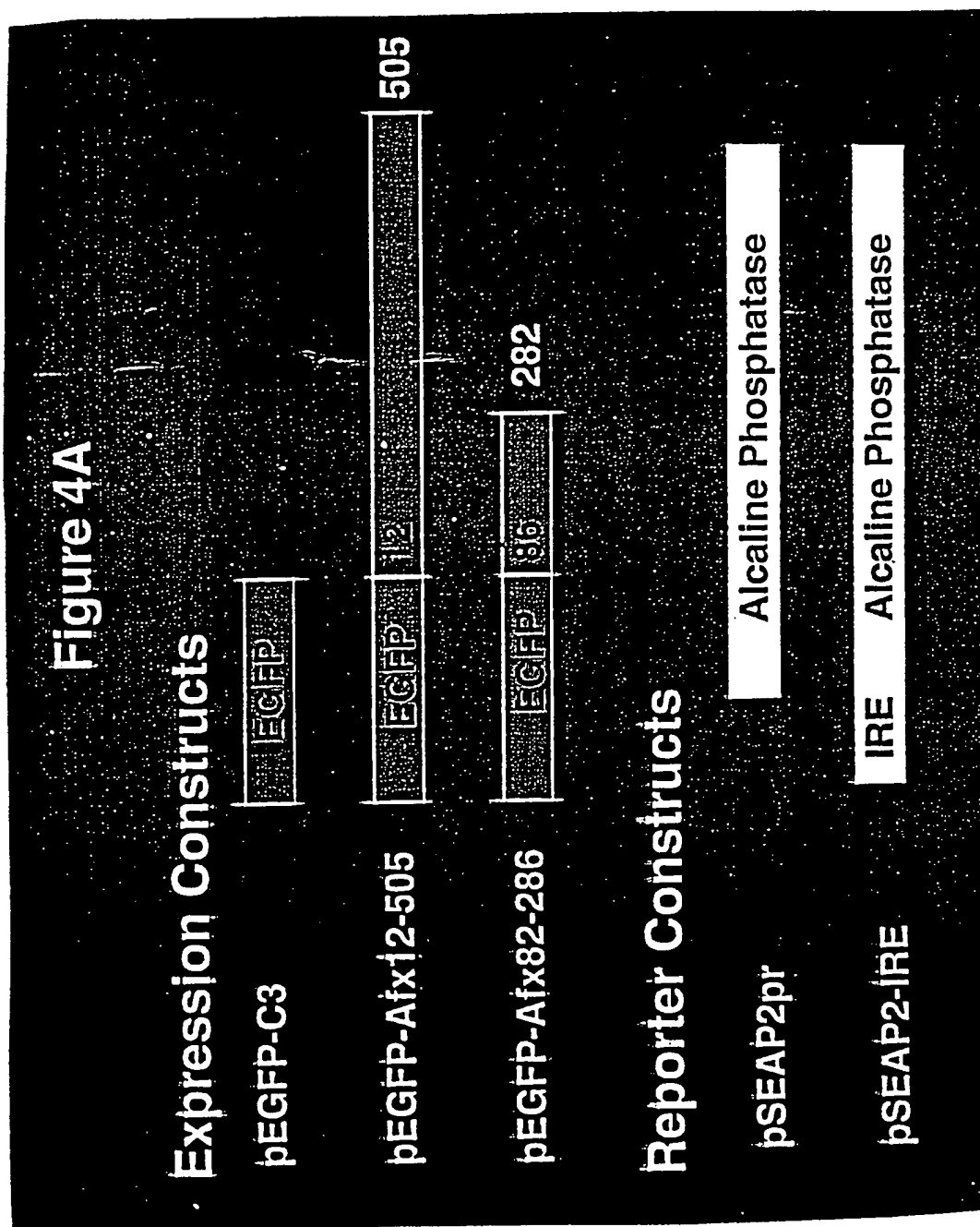


Figure 4A

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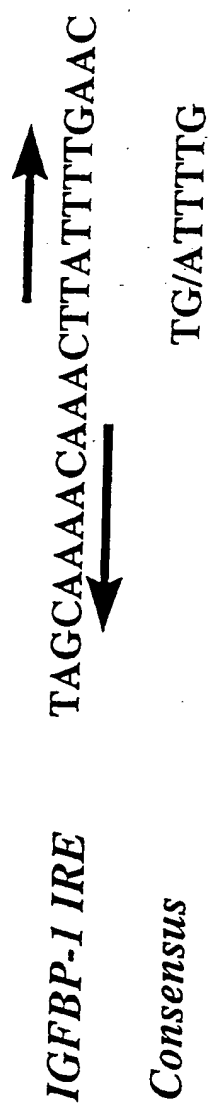


Figure 4B

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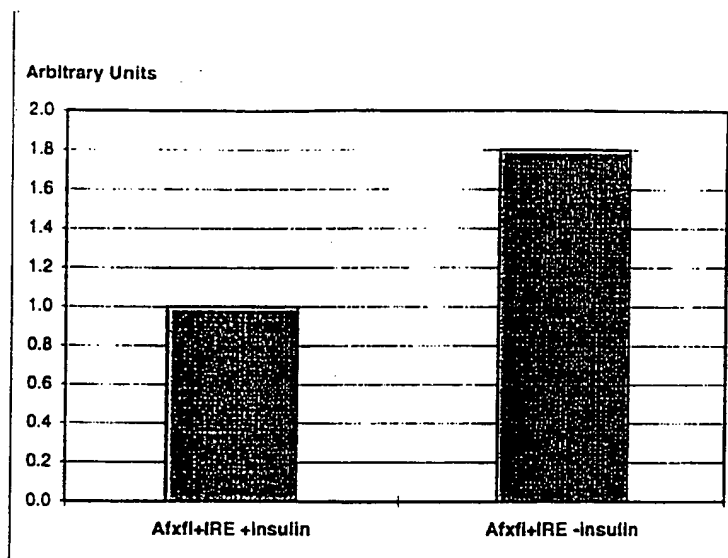


Figure 5

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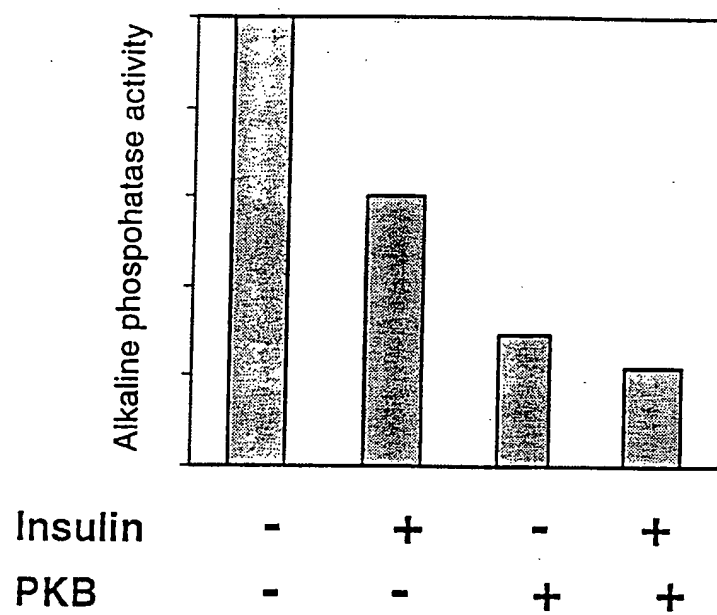


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/02095

A. CLASSIFICATION OF SUBJECT MATTER		
IPC7: C12Q 1/48 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC7: C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Cell, Volume 96, March 1999, Anne Brunet et al, "Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor" page 857 - page 868 --	1-14
P,X	NATURE, Volume 398, April 1999, Geert J.P.L. Kops et al, "Direct control of the Forkhead transcription factor AFX by protein kinase B" page 630 - page 634 --	1-14
X	Genes & Development, Volume 12, 1998, Suzanne Paradis et al, "Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 P13 kinase to the DAF-16 transcription factor", page 2488 - page 2498, figure 3 --	1-14
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
24 February 2000		13-03-2000
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer Hampus Rystedt/Els Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 99/02095

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NATURE, Volume 389, October 1997, Scott Ogg et al, "The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans", page 994 - page 999, figure 2d, figure 4, abstract</p> <p>-- -----</p>	1-14